IDENTIFICATION AND CHARACTERIZATION OF A MUTATED GENE AFFECTING NPC ASSEMBLY

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

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The nuclear pore complex (NPC) is a gateway that controls what is exchanged across the nuclear envelope. Therefore, when something affects its assembly, the cell will die. The laboratory has a collection of *Saccharomyces cerevisiae* mutant strains with NPC assembly defects, but where these defects are located and how they are related to NPC assembly remains unknown. The goal of this project is to identify the mutated gene that is causing assembly defects within one of the strains. Fluorescent microscopy was used to confirm the presence of a NPC assembly defect, and the growth rate of the mutant strain was measured at the permissive and nonpermissive temperature. Additionally, a tiled genomic library was used to compliment the temperature sensitive defect as well as NPC assembly defect. Fluorescent microscopy confirmed the presence of an assembly defect. It was additionally found that the growth rate of the mutant was significantly slower at the nonpermissive temperature. Upon completing all transformations, none of the plasmids in the genomic library rescued the mutant. With information still missing by the mechanism of which NPC's are formed, identifying and characterizing genes that affect NPC assembly can lead to more information about it.

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NOMENCLATURE

NPC	Nuclear Pore Complex
GFP	Green Fluorescent Protein
Nup	Nucleoporins
npa	nuclear pore assembly

CHAPTER I

INTRODUCTION

The Nuclear Pore Complex

The nuclear pore complex (NPC) is a pore that spans the nuclear envelope and allows bidirectional transport of molecules between the nucleus and the cytoplasm. In addition to letting these molecules pass, the NPCs prevent dangerous and unwanted molecules from entering the nucleus. When NPC assembly is disrupted, the cell will die (Schwarts, 2016). By regulating nuclear transport, the NPC plays a major role in gene expression, cell signaling, and cell growth. The NPC is also thought to play a role in cell division, including chromatin silencing. All of these processes are essential for proper cell functioning, making the NPC an important contributor to cellular life (Suntharalingam and Wente, 2003).

The NPC's are made of proteins known as nucleoporins (Beck and Hurt, 2016). Approximately 30 different nucleoporins have been found, and each NPC contains 500 or more individual nucleoporins (Schwarts, 2016). Approximately 20 of these nucleoporins have been found to be conserved among eukaryotes (Schwarts, 2016). NPCs measure approximately 44-50 MDa, approximately 100 nm in diameter, and approximately 80 nm in length (Knockenhauer and Schwartz, 2016). Through electron microscopy, the channel itself has been found to measure around 37 nm in diameter (Alber et. al, 2007). The core of the NPC consists of a multiple of eight "spokes" that are situated in the nuclear pore, and this arrangement appears to be universal across all eukaryotic species. The spoke is further divided into asymmetrical nuclear basket and cytoplasmic filaments (Hayama et. al, 2017). The spokes and Nups form two outer rings and two

inner rings within the pore. The outer and inner rings form the core scaffold, the center of the pore that allows for bidirectional transport (Upla et. al, 2017).

NPC Assembly

Although there is plenty of information about NPCs, there is not as much information known about NPC assembly. Identifying genes that affect nuclear pore complex assembly, or the nuclear pore complex itself, provide more information about this vital process in every eukaryotic organism. In many eukaryotes, the NPC is known to disassemble during nuclear envelope breakdown during mitotic division. As the nuclear envelope reforms during telophase, nuclear membrane vesicles bind to the newly separated chromatin. It has been further found that Ran GTPase, a protein involved in the transport of molecules through the NPCs, is an essential part of nuclear envelope assembly in higher eukaryotes. This conclusion has been confirmed by experiments that add Ran GTPase inhibitors during assembly and observing abnormal fusion patterns. The precise details on nuclear envelope reformation after mitosis are still unclear, making it an active area of research (Hetzer et. al. 2000). Similarly, there are still many details missing from the process of NPC assembly after Mitosis. In 2003, it was found that Ran GTPase facilitates Nup associations and signals NPC insertion. This interesting discovery provides an explanation as to how the NPC inserts itself into the nuclear membrane after reassembly (Walther et. al. 2003).

In yeast and other lower level eukaryotes, the process of NPC assembly is different due to variations in mitosis. The nuclear envelope of yeast cells does not disassemble during mitosis like most other eukaryotes. This process of closed mitosis requires that NPC assembly occur in an already formed nuclear membrane. It is thought that NPC assembly in yeast occurs by localization and association of Nups with integral membrane proteins, such as the Ran GTPase.

This will cause association between the outer and inner nuclear membrane to which the new pore can form. Upon fusion with the membrane, more Nup sub-complexes are inserted into the pore until the NPC is fully assembled (Suntharalingam and Wente, 2002). While there are still details of the process that remain unclear, NPC assembly in yeast is thought to occur by fusion of nuclear vesicles. This is followed by the fusion of the inner and outer membrane in an area in which the pore forms. Similar to higher eukaryotes, there are still several elements of NPC assembly that remain unknown (Ryan et al. 2003).

GFP-Nup Patterns

Since NPC assembly is essential for the NPC's characteristic bidirectional transport, a bank of yeast with temperature sensitive defects was generated and screened for mutations (Ryan and Wente, 2002). By fusing green fluorescent protein (GFP) to specific Nups, the phenotypes expressed by the yeast were able to be thoroughly examined. It was predicted that yeast that had wild-type NPC's would show a more organized structure than the NPC's of the mutants. Indeed, the wild-type cells shows a bright and continuous GFP pattern around the nucleus, indicating normal assembly and functioning nuclear pore assembly (*npa*) genes. Meanwhile the mutant strain is characterized by mislocalization and discontinuous GFP patterns about the nucleus. At room temperature (the permissive temperature), the mutants show small growth phenotype compared to the wildtype. However, upon moving to a nonpermissive temperature, the mutants grow minimally. A yeast genomic library (collection of genomic DNA from yeast) was used to rescue the mutation in order to learn more about what genes affect assembly. These differences in the GFP patterns between the wildtype and the mutant strains allow for identification of mutated genes that affect the NPC and its assembly (Ryan and Wente, 2002).

Due to the fact that the yeast genome has been well studied, several genomic libraries have been produced to conduct research on gene functions. An example of one of these genomic libraries is the Prelich library. The Prelich library is a tiled genomic library that contains 1,588 plasmids divided into 17 different pools. The Prelich library accounts for more than 95% of the yeast genome (Jones, Prelich et. al 2008). The wide coverage of this library makes it a valuable resource in complementation. However, more than 300 genes (approximately 4.5%) are still missing from this library. Despite this, the library was found to be advantageous for systematic overexpression screens (Jones, Prelich et. al 2008).

Using a mutant from the bank mentioned earlier, my project will focus on the identification of the gene causing a mutated phenotype in the NPC. This question is very similar to the research done in the experiments. My project is different because it will address a different gene, but I will use similar materials and methods to achieve an answer to the research question. By using mutants that are temperature sensitive in addition to having mislocalized NPC's, a wildtype version of the gene can be inserted into it from a genomic library. After the correct wildtype gene is found, genomic sequencing can be done to identify the gene of interest. By completing this project and identifying the mutated gene, more information can be obtained about nuclear pore assembly.

CHAPTER II

METHODS

Measuring the Growth Rate of the Wild-type and Mutant Strains

A mutant supplied by the lab, *KRY141*, was compared to the wild-type strain by visualizing their respective GFP-Nup patterns. The GFP-Nup patterns of the wildtype, *YGS53*, were compared to *KRY141* using fluorescent microscopy at the 100X objective. The growth rate of *KRY141* was measured by inoculation into 50 mL of YPD, and the cell density was measured over time using a spectrophotometer set at 600 nm. The growth rate of *KRY141* and *YGS53* were measured at both the permissive and nonpermissive temperature three different times in order to determine the doubling rate. The data was graphed using Excel in order to find an equation for the best fit line and the standard deviation. This line was graphed in order to display the growth rate of the strains.

Complementation of *KRY141* with Genomic Library

Complementation of the mutant phenotypes was done through a series of transformations. Therefore, the mutant was grown in YPD until the logarithmic growth phase. Upon harvesting the yeast, transformations with the genomic library were performed using a standard Lithium Acetate Transformation procedure (Ito et. al. 1983). After the transformation, the *S. cerevisiae* were plated on –Leu plates (plates lacking the amino acid leucine). These plates ensured that only colonies that took up a plasmid continued to grow. One plate was left to grow at room temperature (23°C) while the others were shifted to a nonpermissive temperature (34°C). After allowing time for growth, the colony counts were done to assess whether the plasmid rescued the phenotype. The colony count for each pool in the Prelich library was determined in order to

calculate the probability of error when screening for the plasmid (Jones, Prelich et. al 2008). The number of colonies that grew at the permissive temperature were used to estimate the colonies that should have been on the plates at the nonpermissive temperature.

CHAPTER III

RESULTS

NPC Assembly and Growth Characterization

GFP-Nup visualization for the wildtype and mutant was done using fluorescence microscopy in order to confirm the presence of a mutant *npa* gene in *KRY141*. Observations from the fluorescent microscopy allowed for a comparison to be made between the two strains and allowed for the description of the GFP-Nup pattern of the mutant. The wildtype and the mutant strain were grown to early logarithmic phase at the permissive temperature and shifted to the nonpermissive temperature for about five hours. The DIC setting was used to capture the overall morphology while the fluorescent setting was used to capture the GFP-Nup pattern.

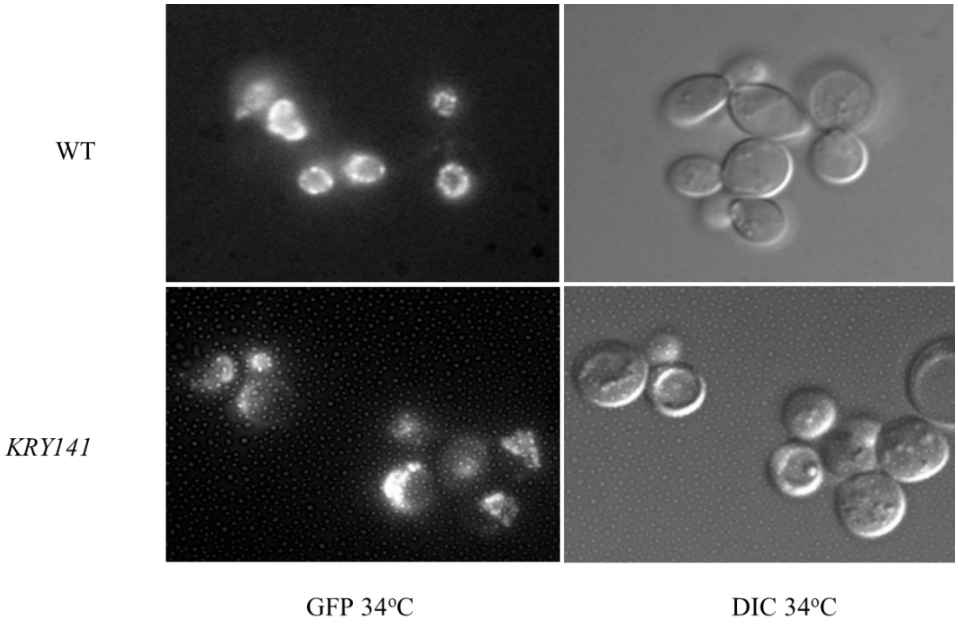

Figure 1. GFP-Nups show a characteristic pattern in the wildtype (*YGS53*) while *KRY141* shows clear mislocalizations.

As seen in Figure 1, the wild-type cells show a punctate and continuous GFP-Nup pattern around the nucleus, indicating that the NPCs were assembled correctly. Unlike the pattern of *YGS53*, *KRY141* shows GFP-Nup patterns that are scattered and disorganized, indicating that NPC assembly was not completed correctly. This disorganized pattern confirms that the *KRY141* strain does have a defect in nuclear pore assembly.

To determine the doubling time and to measure the temperature sensitivity of the *npa* defect, the growth rates of both strains were measured at 23°C and 34°C over a nine hour period. Doubling times of the wild-type and mutant strains were found at room temperature in order to establish their normal growth rate. The growth rates at room temperature were later used in comparison with the growth rates at the nonpermissive temperature.

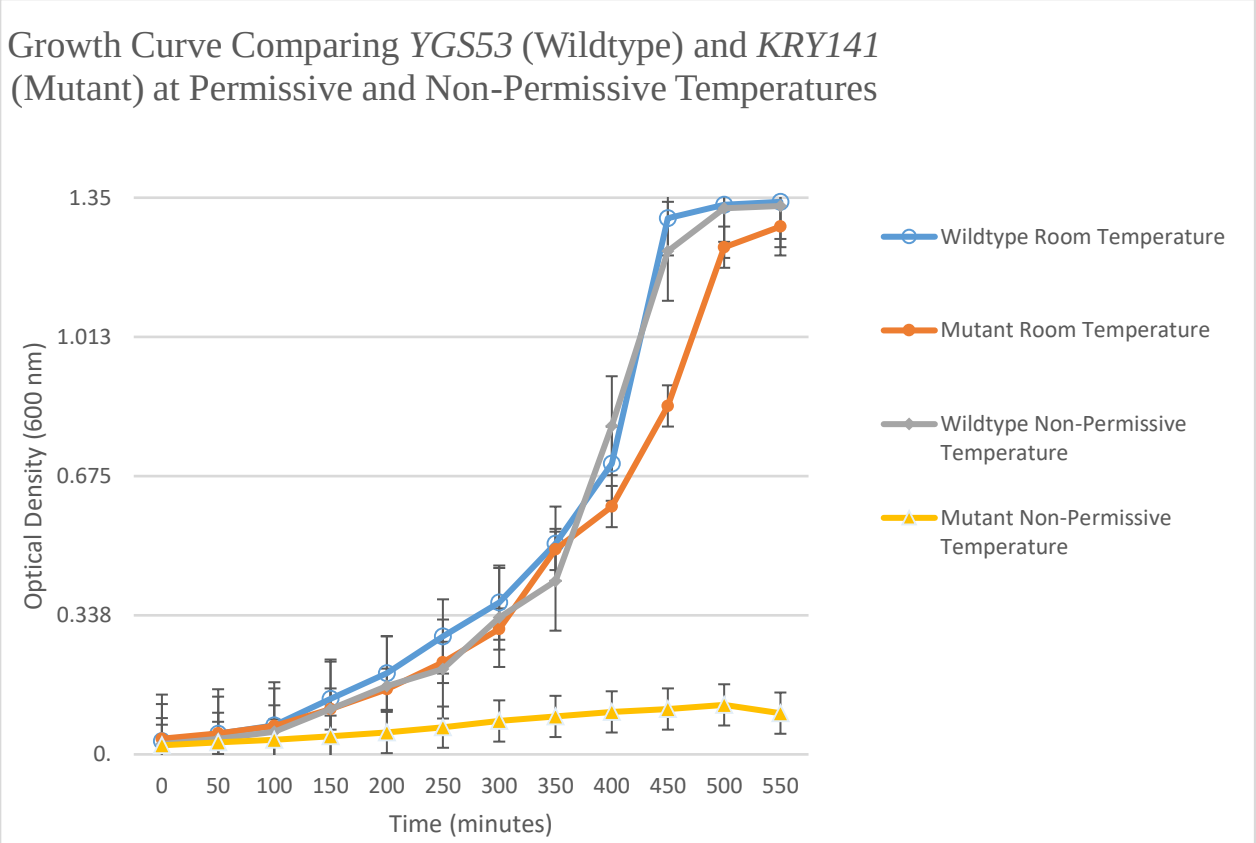

Figure 2. Growth Rate of *KRY141* and *YGS53* at 23°C and 34°C

Table 1. Doubling Times and Standard Deviations for Each Strand at 23°C and 34°C

Strain	Doubling Time 23°C (minutes)	Standard Deviation (minutes)	Doubling Time 34°C (minutes)	Standard Deviation (minutes)
YGS53	90	±5	91	±4
KRY141	105	±4	211	±8

After measuring the growth rates in three different trials, the doubling time of *KRY141* was calculated to be 105 minutes, which is slower compared to the wildtype's doubling time of 90 minutes. The same procedure was carried out for the two strains at the nonpermissive temperature. While there was not a significant difference in the wildtype's doubling time at the different temperatures, *KRY141* showed dramatic differences in the growth rate. As seen in Table 1, the doubling time of *KRY141* at the nonpermissive temperature is 210 minutes, which is slightly over 3 hours. Additionally, Figure 2 shows that *KRY141* only experienced two doubling times before trailing off after nine hours of growth, implying that growth was seriously haltered. This growth rate contrasts significantly to the other growth rates which doubled five times during the nine hour period. This difference in growth rate signifies that the *npa* mutant of *KRY141* is temperature sensitive and will fail to adequately grow when shifted to the nonpermissive temperature.

Genomic Complementation to Identify the Mutant Gene

In an attempt to identify the gene affecting NPC assembly, complementation of the temperature sensitive mutant phenotype was done through a series of transformations using pools from the Prelich library (Jones, Prelich et. al 2008). Upon finding a plasmid that complemented the phenotype, the plasmid could be sequenced to identify the mutant *npa* gene. One of the plates of transformed cells was kept at room temperature to grow to assess how many should have been seen at 34°C. Meanwhile, the two other plates were shifted to the nonpermissive temperature to

select for rescue. After completing the transformations, it was found that none of the plasmids in this tiled genomic library successfully rescued the temperature sensitive defect that *KRY141* displayed. Due to the fact that none of the pools rescued the mutant, Poisson's Distribution was used to calculate the probability that the plasmid was not taken up. To determine P(0), first, the number of colonies were counted at room temperature then multiplied by two in order to account for the fact that two plates were shifted to the nonpermissive temperature per transformation. This number was then divided by 96, the number of plasmids in each pool. Doing this division calculated the average number of times each plasmid in the pool was screened, as seen in Table 2[a]. Finally, the average was used to calculate the P(0) for each pool.

Table 2. Results of Complementation Using Tiled Genomic Library

Pool	Colonies at 23°C	Colonies Screened at 34°C	Average Times Each Plasmid in Pool Screened[a]	Colonies at 34°C	Probability of Missed Plasmid (P(0))
1	961	1922	20.02	0	2.02×10^{-9}
2	586	1172	12.21	0	4.9×10^{-6}
3	595	1190	12.39	0	$4.2x\ 10^{-6}$
4	373	740	7.71	0	$4.4x\ 10^{-4}$
5	592	1184	12.33	0	$4.4x\ 10^{-6}$
6	638	1276	13.29	0	1.7×10^{-6}
7	466	932	9.71	0	6.1×10^{-5}
8	602	1204	12.54	0	3.6×10^{-6}
9	493	986	10.27	0	3.5×10^{-5}
10	750	1500	15.63	0	1.6×10^{-7}
11	631	1262	13.15	0	1.9×10^{-6}
12	542	1084	11.29	0	1.2×10^{-5}
13	310	620	6.46	0	1.6×10^{-3}
14	502	1004	10.46	0	2.9×10^{-5}
15	445	890	9.27	0	9.4×10^{-5}
16	793	1586	16.52	0	6.7×10^{-8}
17	489	978	10.19	0	3.8×10^{-5}

CHAPTER IV

CONCLUSION

NPC Assembly and Growth Characterization

The use of fluorescent microscopy allowed for the visualization and comparison of the wild-type and mutant strains. The GFP-Nup patterns of the wildtype versus *KRY141* are as expected, given that the wildtype had its characteristic punctate pattern and the mutant had a severely mislocalized pattern (Figure 1). This severally mislocalized pattern confirms the NPC assembly defect in *KRY141*. Given that there is still missing information about the assembly process, there are several possibilities that this gene could encode for. By attempting to identify the gene that is affecting NPC assembly, more can be learned about the assembly process.

As seen in Table 1, the slower doubling time between *KRY141* and *YGS53* at the nonpermissive temperature supports the fact that the *npa* defect is temperature sensitive. The temperature sensitivity of the *npa* defect is also supported by the fact that the growth of *KRY141* began to stop after two doubling times, as seen in Figure 2. This halt in growth indicates that the mutant can no longer survive at this temperature. This conclusion is not surprising given the importance of NPCs and their proper assembly. The slower doubling time could result from the fact that important proteins, mRNA, and other essential cell components are impeded when crossing the nuclear membrane if the NPCs are not assembled correctly. The transport of these molecules to and from the nucleus are essential to adequate cell growth and survival. As the mediator of bidirectional transport between the cytoplasm and nucleus, the NPCs play an important role in managing the transport of these macromolecules. If proteins and mRNA

essential to the cell's survival are not able to be efficiently transported, the cell's doubling time will increase.

Genomic Complementation

As seen from Table 2, none of the plasmids in the Prelich library were able to successfully rescue the temperature sensitive mutant phenotype (Jones, Prelich et. al 2008). Poisson's Distribution shows the probability that the plasmid was missed upon transformation. All probabilities are found to be incredibly small which signifies that missing the plasmid was not likely due to faulty technique or inadequate transformations, rather the correct plasmid was not present. The only P (0) that is slightly higher than the others is the probability of Pool 13. However, this is still less than a 0.1% chance that the plasmid was simply missed, strongly implying that the plasmid is not within that pool. The rest of the results of Poisson's Distribution are even smaller, indicating that it is highly unlikely that the plasmid was missed. While the possibility exists that the gene is contained in the Prelich library, Table 2 shows that the probability that this plasmid is present in the library is incredibly low. Therefore, it is nearly certain this genomic library does not contain the correct plasmid.

The Prelich library, an ordered, tiled library, lacks 300 genes, therefore, it is not unreasonable that none of the plasmids from the library were able to rescue the mutant. There is a possibility that the mutated gene of *KRY141* could be one of these missing genes, especially since the probability that is was missed in the library pools is so low. While it is also possible that other genomic libraries could be used to complement this mutation, two other genomic libraries have been screened in previous projects with no successful results. Therefore, the next course of action to successfully identify the mutation would be to sequence the entire genome of *KRY141* and identify the mutation from there. Sequencing the mutant will allow for the

identification of the gene that is affecting NPC assembly. Upon identification of the mutated gene, further experiments can be developed in order to understand that gene's purpose in NPC assembly. Given that there are still several unknown steps in the process of NPC assembly, identification of this gene should give the scientific community more insight.

Using mutant strains with a defect involving NPC assembly allows for a more comprehensive understanding of the genes essential to this important process upon their identification. Identification of genes that play a role in NPC assembly allow for more information to be discovered about this vital process.

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